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Hypo-phosphorylation leads to nuclear retention of NF-κB p65 due to impaired IκBα gene synthesis

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Abstract **Subcellular localization guided by IκBα is crucial for regulation of nuclear factor-κB function. Here, we show that p65 Rel homology domain phosphorylation mutants are transported into the nucleus after IκBα degradation, but as a consequence of lower IκBα levels their relocation to the cytosol is blocked. We demonstrate that phosphorylation of residues S205, S276, and S281 of p65 is not required for interaction between p65 and IκBα, but is pivotal for regulating cellular IκBα levels by positively affecting gene synthesis. Our findings indicate that reduction of phosphorylation leads to nuclear retention of p65, which might be partly responsible for altered transcriptional behavior of p65 serine mutants.**

Keywords: NF-κB; IκBα; Protein phosphorylation; Transcriptional activity; Nuclear localization

1. Introduction

Nuclear factor-κB (NF-κB) is a key regulator of inflammatory and immune responses [1]. Pathological conditions including inflammation and cancer are linked to dysfunction of NF-κB, therefore a tight regulation of its activity is mandatory [2]. The mammalian NF-κB family consists of five members, NF-κB1 (p50), NF-κB2 (p52), c-Rel, RelA (p65), and RelB, which all can form homo- or heterodimers, the p50/p65 heterodimer being the predominant form in most cell types. All NF-κB subunits contain a N-terminal Rel homology domain (RHD), which confers DNA-binding, dimerization, and inhibitor-binding activity, but only p65, RelB, and c-Rel contain a potent C-terminal transcription-activation domain (TADs) and can therefore activate transcription.

NF-κB activity is controlled by its intracellular localization, which is achieved by the family of inhibitor of NF-κB (IκB) proteins. In resting cells, the NF-κB dimer forms a complex with IκBα, which masks its nuclear localization signal (NLS) thereby preventing nuclear translocation [3]. Upon activation, IκBα is phosphorylated by the IκB kinase (IKK) complex, leading to rapid ubiquitination and proteolysis via the 26S proteasome [4,5]. This results in nuclear import of NF-κB dimers and transcription of target genes, among them IκBα [6], which is part of a negative feedback loop indispensable for terminating the activity of NF-κB. Newly synthesized IκBα translocates to the nucleus, where it binds to NF-κB, inhibiting its DNA-binding and transporting it out of the nucleus [7]. Therefore, IκBα is crucial for ensuring a rapid and transient NF-κB transcriptional response.

In addition to nuclear translocation, post-translational modifications, particularly phosphorylation, are critical for full activation of NF-κB. Both the RHD and the TAD of p65 contain key phospho-acceptor sites that are specifically targeted by different kinases [8]. p65 transcriptional activity is markedly increased through phosphorylation of S529 and S536, both located within the C-terminal TAD [9,10]. Differential phosphorylation of p65 at S276 and S311 in the RHD influences p65 DNA-binding and oligomerization properties, as well as its association with CREB-binding protein (CBP)/p300 [11,12]. We have previously shown that the p65 subunit of NF-κB is phosphorylated on multiple residues within the RHD and that phosphorylation at S205, S276, and S281 is essential to retain p65 transcriptional activity [13].

In this work, we investigate the consequences of hypo-phosphorylation of p65 on its subcellular localization. We show that p65 phospho-serine mutants S205A, S276A, and S281A exhibit abnormal localization, which is a consequence of defective IκBα synthesis.

2. Materials and methods

2.1. Plasmids

Episomal and retroviral vectors expressing human p65 and p65 mutants have been described [13]. HA-IκBα was obtained by transferring a BamHI/EcoRI fragment from pKSII/ECI-6 (kind gift from Rainer de Martin, Medical University of Vienna, Austria) to a modified pcDNA3 vector harboring a N-terminal HA-tag.

2.2. Cell culture

HEK293 and RelA$^{-/-}$ mouse embryonic fibroblasts (MEF) [14] were cultured as described [13] and transiently transfected with calcium phosphate or Lipofectamine (Invitrogen), respectively. For

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Abbreviations: A, alanine; CBP, CREB-binding protein; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; HEK, human embryonic kidney; IκB, inhibitor of NF-κB; IKK, IκB kinase; K, lysine; MEF, mouse embryonic fibroblast; NF-κB, nuclear factor-κB; NLS, nuclear localization signal; PBS, phosphate-buffered saline; qPCR, real-time quantitative polymerase chain reaction; Rel; reticuloendotheliosis viral oncogene; RHD, Rel homology domain; SDS, sodium dodecyl sulfate; S, serine; TAD, transcription-activation domain; TNF-α, tumor necrosis factor alpha; wt, wild-type

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stimulation, 10 ng/ml human recombinant tumor necrosis factor alpha (TNF-α) (Biosource) was used. RelA$^{-/-}$ MEF lines stably expressing p65 wild-type (wt) or mutants were developed by exposing cells to retrovirus-containing supernatants as described elsewhere [13].

2.3. Immunofluorescence

MEF were fixed with 3% formaldehyde, permeabilized with 0.5% Triton X-100 and blocked in phosphate-buffered saline (PBS)/0.05% Tween20/0.1% bovine serum albumin (Sigma–Aldrich). Cells were incubated with p65 (C-20; Santa Cruz Biotechnology) and HA antibodies (12CA5; Roche Applied Science) in blocking buffer for 1 h. After incubation with Alexa488-labeled anti-rabbit IgG and/or Alexa568-labeled anti-mouse IgG_{2b} (Molecular Probes) in PBS/0.05% Tween20 for 1 h, they were mounted with SlowFade Gold antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Subcellular distribution of p65 and IκBα was analyzed using a Nikon Eclipse TE2000 microscope equipped with a 40× objective. Pictures were acquired with a charge-coupled device and images were processed with IPLab software (Scanalytics, Vers. 3.9.3).

2.4. Co-immunoprecipitation

Cells were harvested in RIPA buffer containing 0.5% deoxycholate and protease inhibitors (Roche Applied Science) and the resulting homogenate was cleared by centrifugation. The cleared lysate was subjected to immunoprecipitation with either anti-c-myc or anti-HA agarose (Sigma–Aldrich). After 2 h incubation at 4 °C, samples were washed, and bound proteins were eluted in Laemmli buffer. Immunoprecipitated proteins were detected by Western blotting with specific antibodies: c-myc 9E10, HA F-7, IκBα C-21 (Santa Cruz Biotechnology), and β-actin AC-15 (Sigma–Aldrich). Protein bands were quantified with Kodak 1 D V3.6 image software on a Kodak Image Station 2000R. Cellular IκBα levels were calculated relative to β-actin levels. IκBα protein level in resting p65 wt expressing cells was set to 1. Levels of precipitated IκBα were calculated as a fraction of cellular IκBα levels.

2.5. Preparation of nuclear extracts

Cells were lysed in 10 mM Tris–HCl pH 8.0, 320 mM sucrose, 3 mM $CaCl_2$, 2 mM $Mg(OAc)_2$, 0.5% Triton X-100, 5 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol (DTT), protease inhibitors. Nuclei were collected by centrifugation, washed, and lysed in 20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 800 mM NaCl, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 1% Triton X-100, 5 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, protease inhibitors. Nuclear debris was removed by high-speed centrifugation. Western blots were probed with β-actin AC-15 as loading control, and histone H1 FL-219 (Santa Cruz Biotechnology) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon) antibodies to ensure compartmental separation.

2.6. Gene expression analysis

Real-time quantitative polymerase chain reaction (qPCR) was carried out as described [13]. Primers used to amplify a 96-bp fragment specific for the murine IκBα gene are: forward, 5-CAG CTG ACC CTG GAA AAT-3 and reverse, 5-ATA GGG CAG CTC ATC CTC TGT-3. Primers for the housekeeping gene hypoxanthine guanine phosphoribosyl transferase have been described elsewhere [13].

3. Results

3.1. Hypo-phosphorylation leads to nuclear retention of p65

p65 S205A, S276A, and S281A exhibit altered phosphorylation status and transcriptional activity compared to the wt protein [13]. As changes in activity of NF-κB often correlate with altered subcellular distribution, we investigated the cellular localization of p65 wt and mutants. In the absence of stimulus p65 wt is exclusively located in the cytosol, whereas mutants also exhibit a small amount of nuclear staining (Fig. 1A). After 30 min TNF-α treatment, p65 proteins translocate to the nucleus regardless of their phosphorylation status. After 120 min stimulation however, p65 wt is relocated to the cytosol, whereas phospho-mutants are retained in the nucleus. Similarly nuclear extracts prepared from sister cultures showed higher nuclear retention of p65 phospho-mutants (Fig. 1B), with nuclear accumulation more pronounced for p65 S276A and S281A, than for p65 S205A. To exclude any temporal shift in the response to TNF-α, one of the mutants (S281A) was followed up to 360 min when it still showed nuclear localization (Fig. 1C). Thus, mutation of S205, S276, or S281 severely affects p65 nuclear export.

3.2. IκBα binding to p65 is not affected by SA mutations

IκBα is the key molecule regulating NF-κB activity by controlling nuclear import and export [7]. As the binding site for IκBα to p65 was narrowed down to the RHD [15], it seemed possible that mutation of certain residues within the RHD could negatively influence interaction. To investigate this possibility we performed co-immunoprecipitation of p65 wt or mutants with IκBα derived from HEK293 over-expressing both proteins. p65 wt and mutants were equally pulled down by precipitation of IκBα (Fig. 2A). Confirming this result, there was also no difference in co-precipitated IκBα when precipitating p65 wt, S205A, S276A or S281A (Fig. 2B). Moreover, stimulation with TNF-α did not affect interaction between p65 and IκBα. Thus, p65 S205A, S276A, and S281A retain their binding affinity to IκBα.

3.3. Cellular IκBα levels are decreased due to impaired IκBα mRNA synthesis in cells expressing p65 S205A, S276A or S281A

Protein expression levels of IκBα are insensitive to TNF-α treatment when both proteins are over-expressed (Fig. 2) indicating that a physiologic balance of IκBα and NF-κB is not given. Therefore we extended our experiments using either HEK293 only over-expressing p65 or RelA$^{-/-}$ MEF stably expressing p65. p65 proteins were precipitated and endogenous IκBα was detected. Confirming results from over-expression experiments, in HEK293 no differences in IκBα association were observed, however, IκBα protein levels were profoundly affected by expression of mutants compared to the wt protein (Fig. 3A). As a consequence less IκBα was found to be associated with p65 mutants. Similarly, p65 wt expression in MEF induced IκBα protein levels, which was more pronounced after TNF-α stimulation (Fig. 3B). In contrast, p65 mutants failed to enhance post-induction IκBα protein expression. Similar to HEK293, binding affinities of p65 wt and mutants to IκBα remained unaltered as evidenced by an unchanged ratio of p65 associated and total cellular IκBα (Fig. 3B).

Considering the significance of phosphorylation of p65 for NF-κB transcriptional activity, the decreased IκBα levels in cells expressing phospho-serine mutants could emerge from defective transcriptional regulation. To test this hypothesis we stimulated RelA$^{-/-}$ MEF stably expressing either p65 wt or mutants with TNF-α and monitored IκBα mRNA expression by qPCR. Cells transduced with empty vector were used as control. IκBα mRNA expression was readily induced by p65 wt, peaking 90 min after TNF-α stimulation (Fig. 3C). In contrast, p65 phospho-mutants could induce IκBα gene expression only up to 50% of the wt protein, which is consistent with observed IκBα protein levels (Fig. 3B). These results indicate that IκBα mRNA expression is dependent on phosphorylation status of p65.

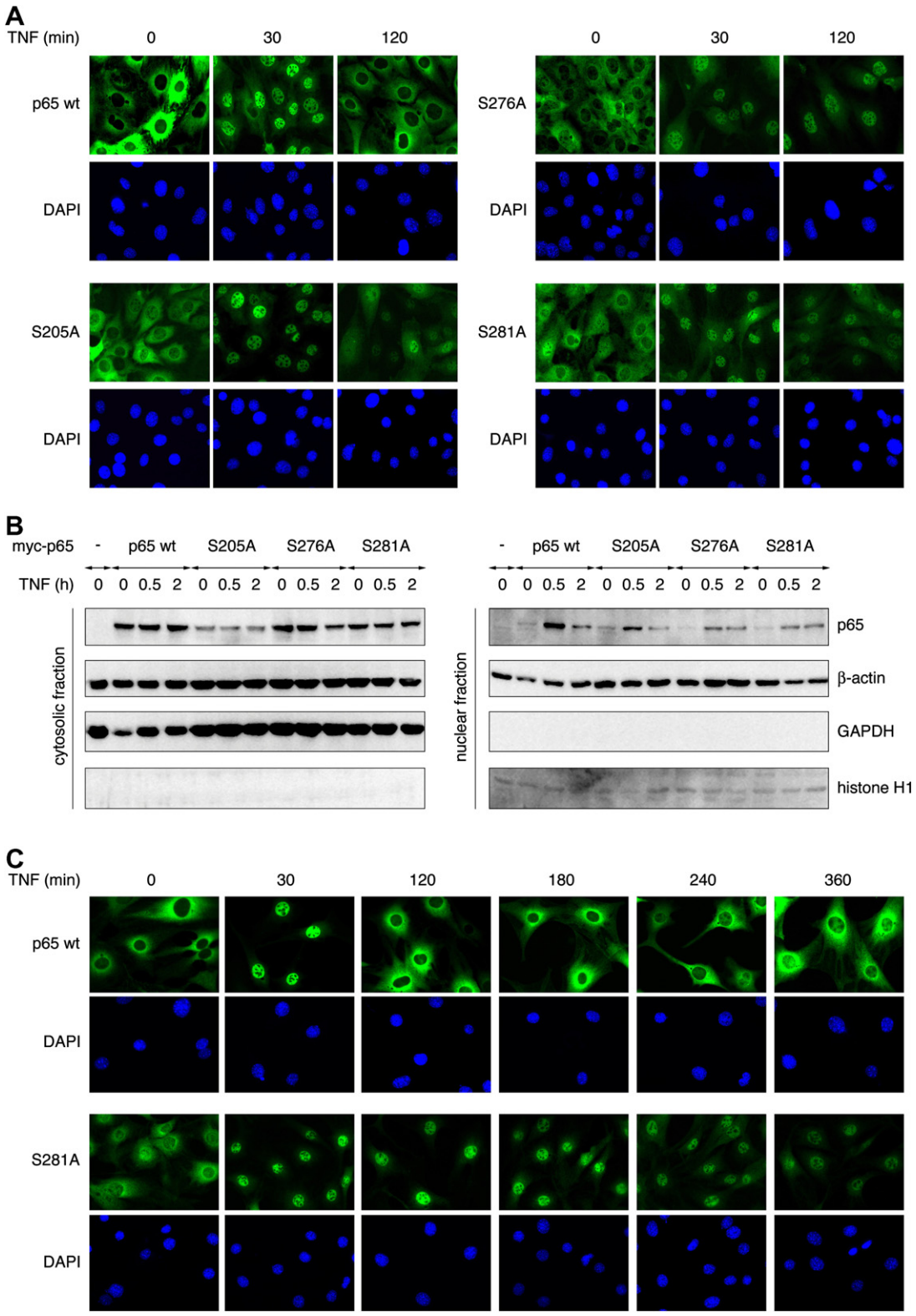

Fig. 1. p65 mutants are preferentially located in the nucleus. (A, C) RelA$^{-/-}$ MEF expressing p65 wt, S205A, S276A, or S281A were treated with TNF-α for indicated time points, and processed for immunostaining with p65 antibody. Nuclei were counterstained with DAPI. (B) Subcellular fractions were analyzed by Western blotting with p65, β-actin, GAPDH, and histone H1 antibodies.

Newly synthesized IκBα translocates to the nucleus and terminates the NF-κB response by binding to NF-κB and shuttling it to the cytoplasm [7]. To determine the amount of nuclear IκBα in p65 mutant-expressing cells we transfected HEK293 with p65 constructs and monitored endogenous IκBα protein expression in cytosolic and nuclear fractions after stimulation with TNF-α for 2 h. As shown in Fig. 3D, nuclear IκBα was barely detectable in cells expressing p65 mutants.

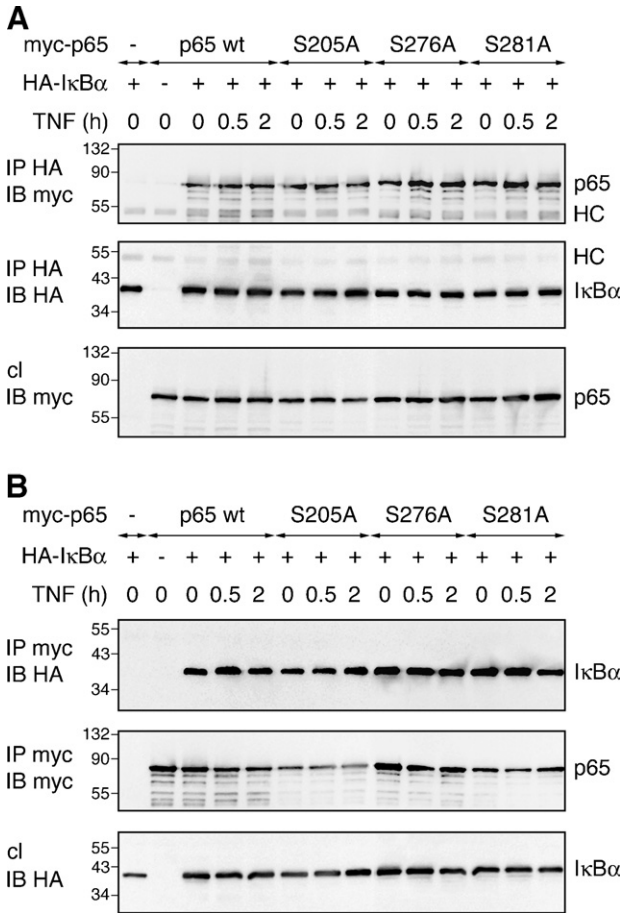

Fig. 2. p65 mutants associate with IκBα. (A,B) HEK293 transfected with p65 and IκBα constructs were stimulated with TNF-α for 0.5 and 2 h. After lysis, cellular extracts were either incubated with HA agarose to precipitate IκBα (A) or c-myc matrix to pull down p65 (B). Co-precipitation of IκBα and p65 was determined by Western blotting using respective antibodies. HC, heavy chain; cl, cell lysate.

While p65 SA mutants lead to 50% reduction in overall IκBα mRNA and protein levels, the effect on nuclear IκBα protein levels seems to be even more significant.

3.4. Re-introduction of IκBα results in restored cytosolic relocation of p65 phospho-mutants

The lack of IκBα protein out of reduced IκBα mRNA production in p65 mutant-expressing cells could account for the observed atypical p65 localization pattern. To prove this hypothesis, we performed indirect immunofluorescence in $RelA^{-/-}$ MEF stably expressing p65 either with or without co-transfection of IκBα. As shown in Fig. 4, re-introduction of IκBα led to cytosolic localization of p65 after 30 min TNF-α stimulation regardless of p65 phosphorylation status. This result finally confirms that the predominant nuclear localization of p65 S205A, S276A, and S281A after TNF-α stimulation is caused by reduced IκBα expression.

4. Discussion

The biological activity of NF-κB is tightly regulated by post-translational modifications and subcellular localization, which is mostly governed by the family of IκB proteins. IκBα is responsible for a strong negative feedback rapidly turning off NF-κB responses and accordingly absence of IκBα results in nuclear accumulation of NF-κB [7,16]. NF-κB/IκBα complexes shuttle continuously between nucleus and cytosol, and dominance of nuclear export over nuclear import and masking the NLS of NF-κB by IκBα contribute to the largely cytosolic localization of NF-κB/IκBα complexes in absence of stimuli [17].

Considering the importance of IκBα in driving p65 localization we contemplated that change in phosphorylation status of p65 could influence interactions between the two proteins and therefore leading to predominant nuclear localization of p65. The crystal structure of NF-κB/IκBα complexes reveal that p65 contacts IκBα through its RHD domain [15], therefore mutations within the RHD could negatively affect binding properties. Although no specific serine residue of p65 was shown to directly contact IκBα, binding between the two proteins is maintained by long-range electrostatic interactions involving charged residues within both proteins [15]. As crystal structures were obtained from bacterially expressed proteins that lack phosphorylation, it was possible that additional charges within p65 RHD introduced by phospho-serines might change the electrostatic surface of p65 and therefore alter IκBα binding.

Phosphorylation has also been shown to regulate acetylation of p65, which in turn can affect IκBα binding [18]. Whereas acetylation of K221 increases DNA-binding affinity and prevents the association of p65 with IκBα, K122/K123 acetylation leads to reduced DNA-binding but facilitates p65-IκBα interaction [19,20]. However, our studies reveal that neither mutation of S205, S276 nor S281 of p65 influences IκBα binding. This result is in line with data published for the Drosophila p65 homologue Dorsal where mutation of six highly conserved serines in the RHD does not eliminate binding of the IκBα homologue Cactus [21].

Having established that p65-IκBα interactions were not changed, we considered the possibility that reduced p65 phosphorylation might negatively influence IκBα gene expression. Indeed, we could show that mutation of potential phospho-acceptor serines within p65 RHD decreases endogenous IκBα protein synthesis after TNF-α stimulation.

This finding is supported by results obtained earlier in our laboratory which showed that transcription from NF-κB DNA-binding elements found in the IκBα promoter is dependent on p65 phosphorylation status [13].

Lack of stimulus-induced IκBα neo-synthesis is responsible for augmented nuclear localization of hypo-phosphorylated p65 species, which however is not associated with increased transcriptional activity. Unlike IκBα binding, alteration in phosphorylation status of p65 could impact binding of other factors, which are not required for nuclear import, but indispensable for transcriptional activation. It was shown that phosphorylation of p65 on S276 promotes enhanced interaction with p300 which accelerates acetylation and transcriptional activity of p65 [18]. In contrast, de-phosphorylation of p65 leads to inactivation of NF-κB by binding to HDAC-1 [22]. Alternatively, cytosolic-nuclear shuttling of p65 might be necessary to maximize NF-κB transcriptional activity by facilitating nuclear IKKα import, which is linked to histone H3 phosphorylation at S10, a modification associated with efficient gene transcription [23].

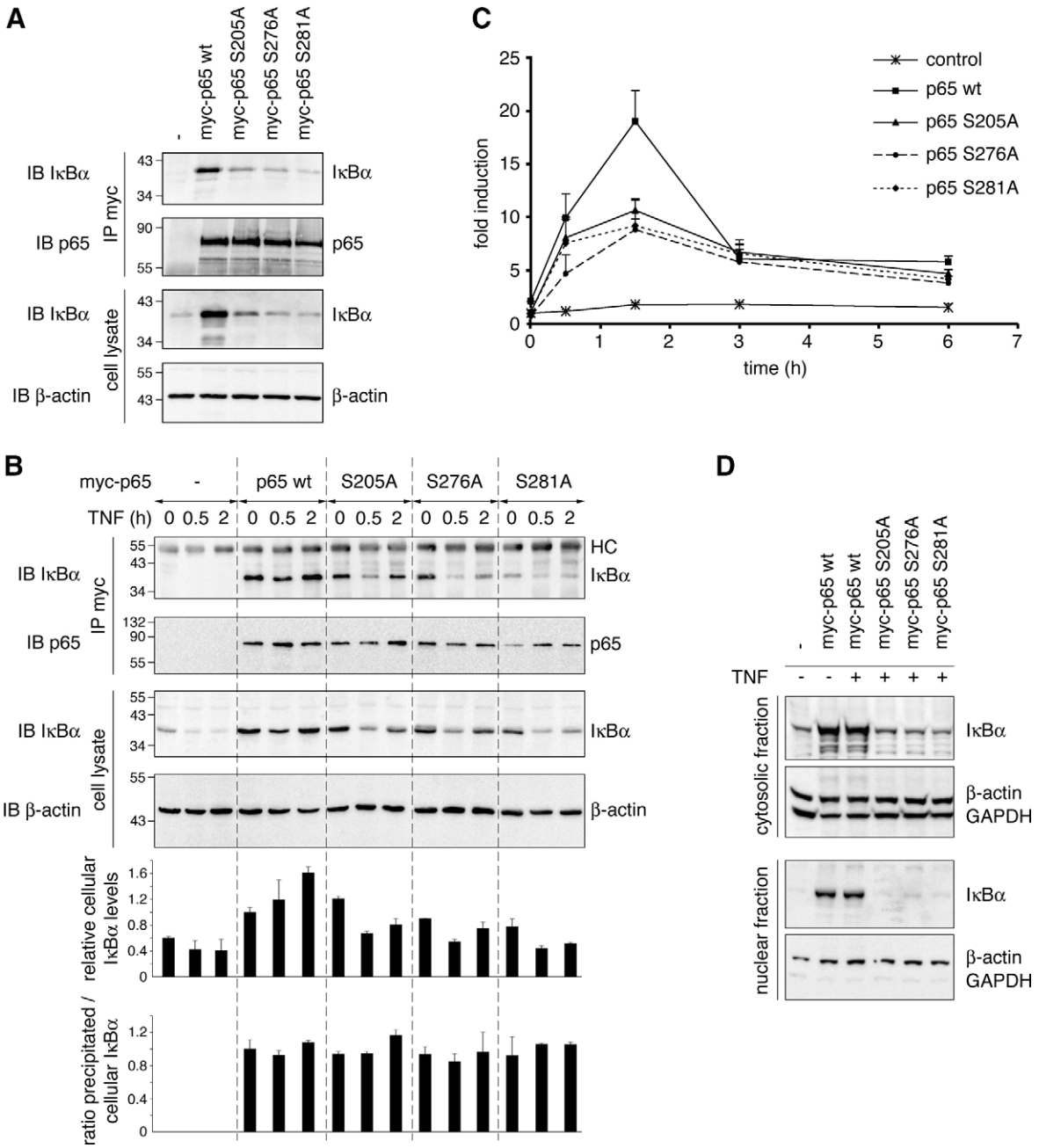

Fig. 3. Impaired IκBα mRNA expression results in lower cellular IκBα protein levels in p65 mutant-expressing cells. (A) HEK293 lysates exogenously expressing p65 wt and mutants were used to pull down endogenous IκBα. (B) RelA$^{-/-}$ MEF reconstituted with p65 wt, S205A, S276A or S281A were stimulated with TNF-α for 0.5 and 2 h. For precipitation of p65 cell extracts were incubated with c-myc agarose and presence of IκBα was determined by Western blotting. Bands in immunoblots were quantified. Error bars represent means ± S.E.M. of two independent experiments. HC, heavy chain. (C) RelA$^{-/-}$ MEF expressing p65 wt or mutants were stimulated with TNF-α for 30, 90, 180, and 360 min, total RNA was isolated, and mRNA levels were analyzed by qPCR. Fold induction of experimental groups was calculated relative to expression level of unstimulated, vector-transfected cells, which was set to 1. Error bars represent means ± S.E.M. of triplicates from three independent experiments. (D) HEK293 were transfected with p65 expression plasmids and stimulated with TNF-α for 2 h. Cell lysates were separated into cytoplasmic and nuclear fractions, which were analyzed by Western blotting for the presence of IκBα. Purity of the nuclear fraction was shown by absence of GAPDH, β-actin was used as loading control for both fractions.

In summary, lack of phosphorylation due to mutations of serines in the RHD of p65 has a great impact on NF-κB transcriptional activity. Our data show that IκBα synthesis is negatively regulated by hypo-phosphorylation of p65. The resulting IκBα deficiency leads to dysregulation of natural cytosolic to nuclear NF-κB balance, which in turn could account for the altered transcriptional activity of hypo-phosphorylated p65. However, the cellular events primarily leading to depleted NF-κB transcriptional activity as a consequence of missing phosphorylation are still unknown and

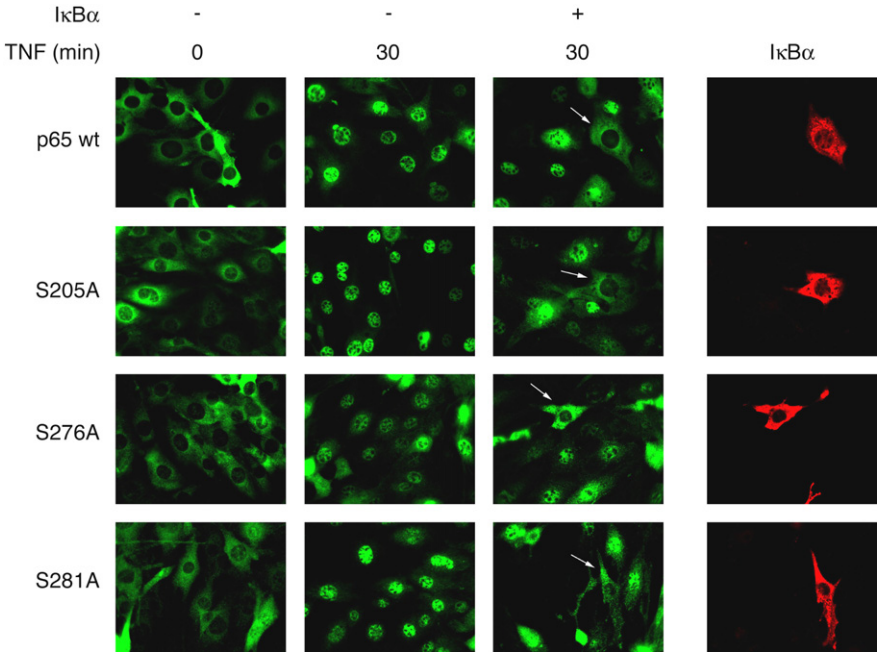

Fig. 4. Reconstitution of IκBα leads to cytosolic relocation of p65 regardless of phosphorylation status. RelA$^{-/-}$ MEF stably expressing p65 wt and mutants were transfected with HA-IκBα and stimulated with TNF-α for 30 min. Cells were immunostained with p65 (green) and HA (red) antibodies. Arrows indicate double-labeled cells.

further investigations will be necessary to identify involved factors.

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